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Ultrastructure and Transport-Related Enzymes of the Gills and Coxal Gland of the Horseshoe Crab *Limulus polyphemus*

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Abstract. The horseshoe crab, *Limulus polyphemus*, may be unique among marine arthropods in that both its book gills and its coxal gland may serve as sites of ion transport. We have therefore examined the ultrastructure of these organs, as well as the distribution and relative levels of two major transport-related enzymes: the $Na^+ + K^+$ ATPase and carbonic anhydrase (CA). The ventral surface of the central region of each lamella shows the typical ultrastructural specializations for ion transport: 10 μm cell thickness, an extensive network of tubules originating from infoldings of the basal membrane, and a high density of mitochondria. This region also contains high levels of activity of the $Na^+ + K^+$ ATPase and CA. The distribution of ion transporting epithelium and transport enzymes is identical in each of the five gill books. The peripheral region of the lamellae of each gill book is specialized for passive gas exchange. The ultrastructural and biochemical profile of the coxal gland is similar to that of the central-ventral region of the gill. *Limulus* possesses the same general mechanism of ion regulation seen in euryhaline decapod crustaceans, but the structural and functional components are uniquely distributed.

Introduction

Euryhaline invertebrates possess a full suite of adaptations for survival in and exploitation of habitats of low and fluctuating salinity. While these adaptations are no doubt common to some extent in all euryhaline marine arthropods, they have been most extensively studied in the decapod crustaceans (*e.g.*, Mantel and Farmer, 1983). Considerably less is known about another arthropod group, the Xiphosura, of which the horseshoe crab, *Limulus polyphemus*, is a member. This investigation focuses on the ultrastructural and biochemical bases of salinity adaptation in *Limulus* as they compare to those in the ecologically similar but more highly derived decapods.

At high salinities, subtidal crustaceans are typical osmotic and ionic conformers. Below a critical salinity, however, many species have the ability to regulate hemolymph osmotic and ionic concentrations above those in the ambient medium. Strong regulators can maintain as much as a 600 mOsmol KgH_2O^{-1} difference between the hemolymph and the surrounding water.

Ion regulation in decapod crustaceans is accomplished primarily through the active uptake of Na and Cl by the gill (*e.g.*, Cameron, 1978). The site of ion transport within the gill is the chloride cell (Foskett and Scheffey, 1982). This cell type is characterized in general as being mitochondria-rich and having an extensive network of infoldings of the basal membrane (Copeland, 1964; Copeland and Fitzjarrell, 1968; Oschman and Berridge, 1971; Ciolfi, 1984). In addition, epithelia that are rich in chloride cells possess high levels of activity of two ion transport-related enzymes, the $Na^+ + K^+$ ATPase and carbonic anhydrase (CA) (Henry, 1984, Towle, 1984).

Chloride cells are heterogenously distributed in the gills of euryhaline marine decapod crustaceans; they are

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more abundant in the posterior gills, especially at low salinities where they form a visible patch in the lamellae (Neufeld *et al.*, 1980; Compere *et al.*, 1989). Consequently, the posterior gills have been characterized as the site of ion regulation, containing dense patches of chloride cells and the associated high levels of transport-related enzymes; the anterior gills are believed to be more specialized for respiratory gas exchange.

The other potential transport epithelium, the antennal gland, is not believed to function in monovalent ion (*i.e.*, NaCl) regulation in euryhaline marine crabs. It produces an isosmotic and isoionic urine at high and low salinities, and it lacks comparable levels of CA activity found in the gill (Cameron, 1978; Cameron and Batterton, 1978; Henry and Cameron, 1982a). As a result, the antennal gland is thought to function in divalent ion regulation and to rid the extracellular compartment of excess fluid, with Na and Cl being lost in the bulk flow.

The horseshoe crab, *Limulus polyphemus*, is a marine chelicerate that is also euryhaline. Although much of its adult life is spent in offshore waters of high salinity (35 ‰), adult *Limulus* invades and survives in estuarine waters as low as 7 ‰ during its annual spawning migration (McManus, 1969; Cavanaugh, 1975; Cohen and Brockman, 1983). Characterized as a relatively weak osmotic regulator, the horseshoe crab maintains the osmotic concentration of its hemolymph roughly between 150 and 200 mOsm $KgH_2)^{-1}$ above ambient in low salinity (Robertson, 1970; Towle *et al.*, 1982, Mangum *et al.*, 1986). Although the gills of *Limulus* are not homologous to those of other marine arthropods (*e.g.*, crustaceans), they are functionally analogous. As in other marine arthropods, *Limulus* gills are morphologically differentiated into thin (peripheral) and thick (central) regions that, at least superficially (*i.e.*, gross morphology and tissue thickness), resemble the subdivisions of the respiratory and ion transporting regions, respectively, of crustacean gills. In *Limulus*, however, which possesses typical chelicerate book gills, the thick region is located centrally on each gill lamella, and the thin region is peripheral (Mangum, 1982).

On the other hand, the coxal gland of *Limulus*, which is homologous to the antennal gland of crustaceans, does not appear to be completely analogous in function. In *Limulus* acclimated to low salinity, the coxal gland produces urine that is hypoosmotic and hypoionic to the hemolymph (Mangum *et al.*, 1976; Towle *et al.*, 1982). Both the gills and coxal gland contain measurable levels of $Na^+ + K^+$ ATPase activity (Towle *et al.*, 1982). Therefore, both organs may play an important role in ion regulation. If so, then *Limulus*—one of the few large euryhaline arthropods with a reproductive and life history strategy linked to migration along a salinity gradient—would be set apart from euryhaline decapods in its strategy of low salinity adaptation.

In the present investigation we have compared the differences between two phylogenetically distant, but ecologically similar groups. We present data on the ultrastructural features of the gills and coxal gland that are characteristic of salt-transporting epithelia. The activities of the $Na^+ + K^+$ ATPase and CA in gills and coxal gland were also compared.

Materials and Methods

Collection and maintenance of experimental animals

Adult horseshoe crabs, *Limulus polyphemus* (>20 cm carapace width), were collected from three locations: (1) an oceanside beach in Accomack County on the Eastern Shore of Virginia, (2) the Hampton Roads area of the Chesapeake Bay, and (3) the Gulf of Mexico near Port St. Joe, Florida. Animals were maintained either in running seawater tables (29–32 ‰ salinity) at the Virginia Institute of Marine Science, Eastern Shore Laboratory, Wachapreague, Virginia, or in 25–35 gallon plastic tanks, containing seawater of similar salinity, and equipped with biological filters for long-term acclimation in Williamsburg, Virginia, or Auburn, Alabama.

Animals were acclimated to high salinity (32 ‰) for three weeks. For acclimation to 10 ‰, salinity was decreased slowly (3–5 ‰ per day), and the animals were then held at 10‰ for at least two weeks before use. Salinity was measured either with a conductivity meter (Yellow Springs Inst. model 33) or a hand-held refractometer and adjusted with either artificial sea salt or deionized water. The horseshoe crabs were fed fish and shrimp three times per week but were starved for a period of no longer than one week prior to use in an experiment.

Gill ultrastructure

Lamellae from horseshoe crabs acclimated to either high (32–35 ‰) or low (15–18 ‰) salinity were removed from the gills and immediately placed in fixative (2% gluteraldehyde, 150 m*M* sucrose, 100 m*M* phosphate buffer, pH of 7.2–7.4). Small (2 × 4 mm) rectangular pieces of tissue were cut from both the central and peripheral regions of the lamellae. The ventral and dorsal chitinous layers were then separated, producing four sections: central dorsal (CD), central ventral (CV), peripheral dorsal (PD), and peripheral ventral (PV) (see Fig. 1 for a schematic of the dissection). Gill sections were fixed in fresh gluteraldehyde solution for 2 h followed by three 10-min rinses in phosphate buffer. Tissues were then post-fixed for 2 h in 1% osmium tetraoxide in phosphate buffer and serially dehydrated with acetone. The gill tis-

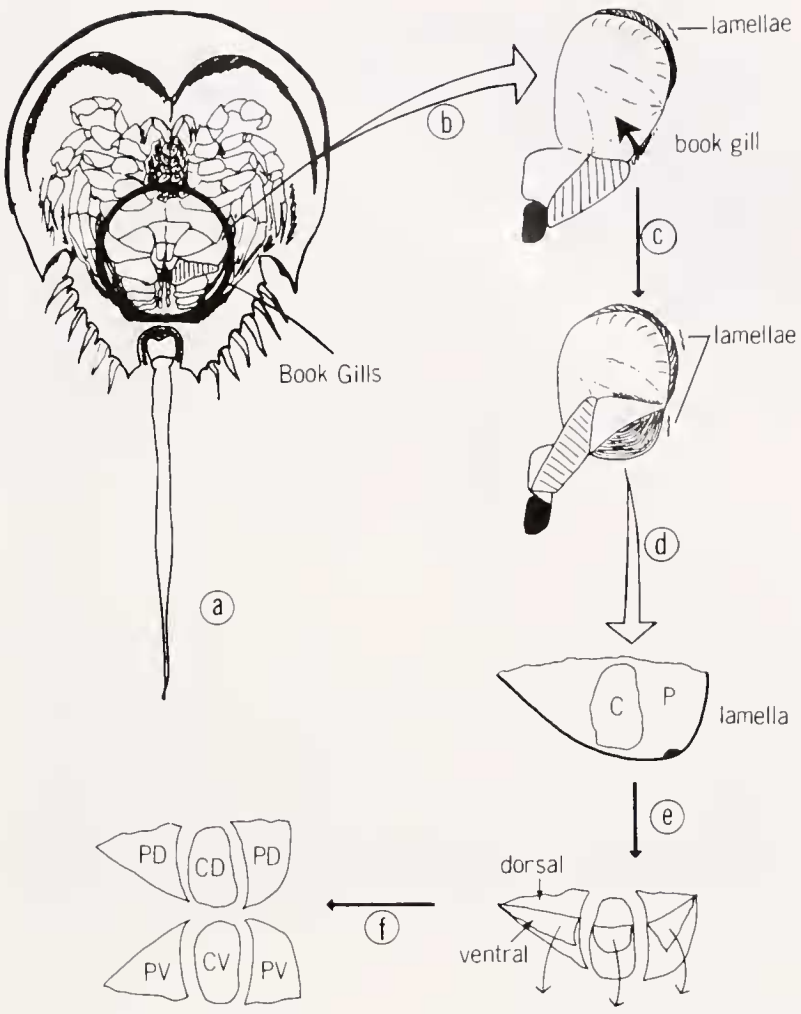


Figure 1. Procedure for dissecting the gill lamellae to produce standardized sections of tissue for electron microscopy and $Na^+ + K^+$ ATPase assays. (a) Ventral view of adult male horseshoe crab; (b) removal of gill book number 1; (c) lifting flap to expose lamellae; (d) removal of individual lamella; (e) removal of thick chitinous edge and dissection of the central from the peripheral regions; (f) separation of ventral from dorsal sides. Resulting regions: PD = peripheral dorsal, PV = peripheral ventral, CD = central dorsal, CV = central ventral.

sue was stained with uranyl acetate for 8–24 h and embedded in either SPURRS or EMBED medium. Thin sections, cut on a Sorvall MT 2-B ultramicrotome, were stained with lead citrate and examined with a Zeiss EM 9S-2 or EM 109 electron microscope.

$Na^+ + K^+$ ATPase activity

Upon the completion of salinity acclimation, all five of the gill books were removed from the animal along with a lobe of the coxal gland. The tissues were placed in about 4 volumes of cold homogenizing medium (0.25 *M* sucrose, 6 m*M* disodium EDTA, 20 *M* imidazole, pH adjusted to 6.8 with acetic acid) (Towle *et al.*, 1982). The central dark patch of tissue was separated from the peripheral region in about 20 lamellae from each gill, and a sample of intact lamellae was taken as well. Tissues were transferred to 20 volumes of fresh homogenization medium containing 0.2 volumes of 10% sodium deoxycholate and homogenized by hand in a ground glass tissue grinder. The homogenate was filtered through two layers of cheesecloth and assayed immediately. Tissue samples from the coxal gland were dissected and homogenized as above without any attempt at separation into anatomically distinct areas.

Total ATPase activity was measured using a PK/LDH linked spectrophotometric assay described by Towle *et al.* (1982). Briefly, the assay measures the oxidation of

NADH at 340 nm that is enzymatically coupled to the the hydrolysis of ATP. The reaction mixture contained 0.1 m*M* NADH, 5 m*M* disodium ATP, 2.5 m*M* PEP, 20 μl PK/LDH, 20 m*M* imidazole (pH 7.8), 90 m*M* NaCl, 10 m*M* KCl, and 5 m*M* $MgCl_2$ in a final volume of 2.0 ml (all chemicals obtained from Sigma, St. Louis, Missouri). After 5 min incubation at 30°C, the reaction was started by the addition of up to 100 μl of filtered homogenate. The change in absorbance was monitored in a temperature-controlled cuvette (30°C) and Bausch and Lomb 700, Beckman DU2 or DK2 recording spectrophotometer. Duplicate assays were run in the presence of 1 m*M* ouabain. The $Na^+ + K^+$ ATPase activity was taken as the difference between the total ATPase activity and the ouabain-sensitive ATPase activity. Total protein concentrations were determined by Coomassie blue dye binding (Bradford, 1976), using dye reagent (Bio Rad) and bovine serum albumin as a standard. Enzyme activity was reported as nanomoles Pi released per mg protein per minute. A two-way ANOVA followed by *a posteriori* testing (LSD) was used to determine significance in enzyme activities among the different gills and between the central and peripheral regions within the gills.

Carbonic anhydrase activity

Gill (central, peripheral, and whole sections) and coxal gland tissue were dissected as above from horseshoe crabs that had been acclimated to either 32 or 10 ‰ salinity. Tissues were placed in five volumes of cold buffer (225 m*M* mannitol, 75 m*M* sucrose, 10 m*M* Tris phosphate, pH adjusted to 7.4 with 10% phosphoric acid) and homogenized with a motor-driven ground glass homogenizer. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C (Sorvall RC-5B), and the supernatant was assayed for CA activity according to an electrometric method described by Henry (1991). Briefly, 100 μl of CO_2-saturated water is added to a reaction mixture containing supernatant and buffer (see above); the initial decrease in pH, due to the rapid hydration of CO_2, is monitored using sensitive pH electrodes and a null-point pH meter. Since the conversion of CO_2 to H^+ has a 1:1 stoichiometry, the change in pH is representative of the rate of CO_2 hydration. Protein concentrations were determined by Coomassie blue dye binding (see above), and CA activities were reported as μmol CO_2 per mg protein per minute. Statistical significance among the anatomical sections of the different gills, or among the whole-gill homogenates, at a given salinity was determined by ANOVA; significance within a single gill at high *vs.* low salinity was measured with Student's *t*-test. A portion of the gonadal operculum, anterior to the gills, was also assayed as a non-epithelial, salinity-insensitive "control" tissue.

Results

Gill and coxal gland ultrastructure

Each page, or lamella, of a book gill contains a thick, dark elliptical patch near its center surrounded by a thinner peripheral region (Fig. 2A). The central patch is similar in appearance to the dark, ion transporting region in the posterior gills of euryhaline crustaceans (*e.g., Callinectes sapidus;* see Neufeld *et al.,* 1980). A longitudinal section through the central region is shown in Figure 2B. A network of pillar cells form channels in the lamellae through which hemolymph is moved; these pillar cells resemble those found in crustacean gill lamellae (Cioffi, 1984). Hemolymph is separated from ambient water by a single epithelial layer on the dorsal and ventral surfaces of the lamellae. The thickness of the epithelium on the central ventral surface (Table I) is typical of ion-transporting epithelia found in gill lamellae of many crustacean species (*e.g.,* Aldridge and Cameron, 1979; see also Henry, 1994, for a recent review). The difference in epithelial thickness between the central ventral and the other lamellar sections appears to be due to the cellular component, since the thickness of the cuticle is the same throughout the gill (Table I). On the dorsal surface the total diffusion distance is only slightly greater (<1 μm) than the cuticle thickness, indicating the presence of a very thin, respiratory type of epithelium. The peripheral ventral region is only slightly thicker, still suggesting an essentially respiratory function.

In contrast, the epithelium from the ventral surface of the central region displays ultrastructural features that are characteristic of salt-transporting cells of arthropod gills. The cells contain an extensive internal network of invaginations, originating from infoldings of the basal membrane (Fig. 3). There is also a very dense population of mitochondria associated with the network of membrane invaginations. At the very apical end of the cell, the membrane network and associated mitochondria are much less dense. Each cell has a prominant nucleus that is located in the basal portion. All of these features are similar to those found in "chloride cells" of both arthropods and fish (*e.g.,* Zadunaisky, 1984). The characteristic features that appear to be lacking in the epithelial cells of the peripheral region are the extensive system of membrane invaginations, the dense population of mitochondria, and the thick cellular component of the epithelium (Fig. 4A *vs.* 4B). Additional details may be found in Jackson (1986).

The coxal gland appears to have the same ultrastructural characteristics correlated with salt transport that are found in the central ventral region of the gills. There is an extensive network of membrane invaginations

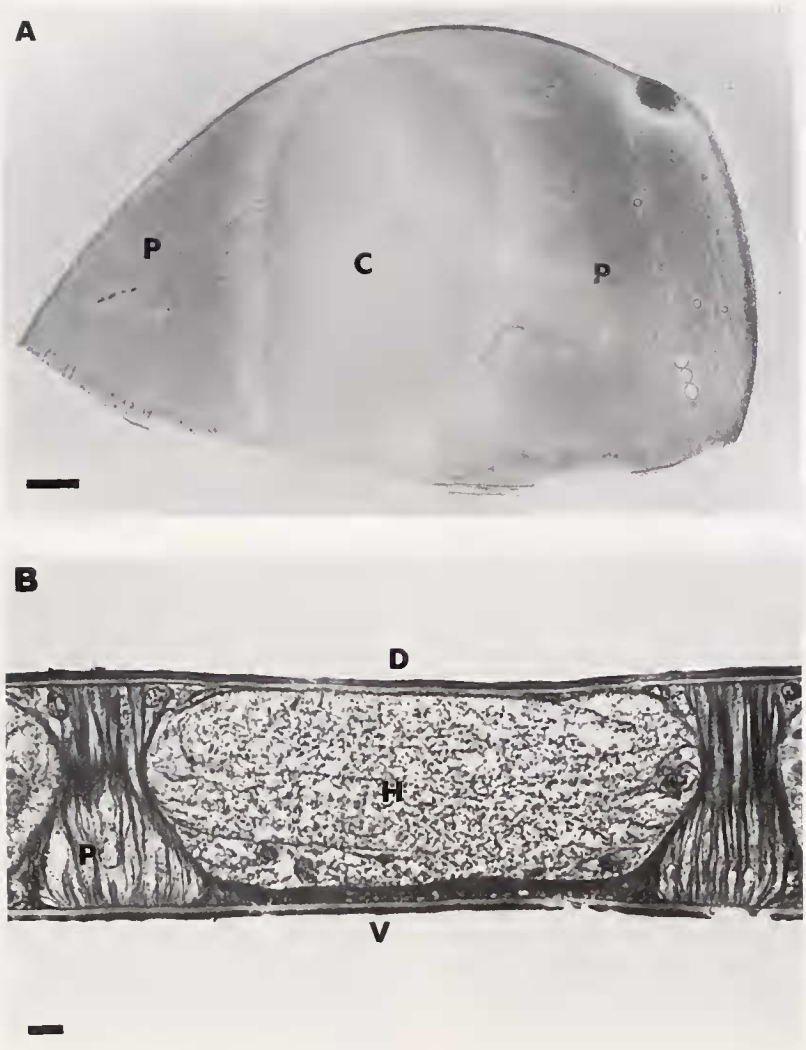


Figure 2. (A) Whole mount of a single lamella showing central (C) and peripheral (P) regions. ×4.5, scale bar = 2 mm. (B) Longitudinal section through the central region showing the thin dorsal (D) and thick ventral (V) epithelial layers. P = supporting pillar cell network. H = hemolymph space. ×608, scale bar = 10 μm.

Table I

Cuticular thickness and hemolymph-water diffusion differences in the various sections of horseshoe crab gills

Section	Cuticle thickness (μm)	Diffusion distance (μm)
CV	3.7 ± .10 (18)	9.1 ± .24 (14)
CD	3.0 ± .02 (8)	3.9 ± .13 (8)
PV	3.4 ± .08 (20)	4.7 ± .31 (10)
PD	3.0 ± .07 (26)	3.9 ± .10 (22)

Values reported as Mean ± SEM (N). CV = central ventral, CD = central dorsal, PV = peripheral ventral, PD = peripheral dorsal.

throughout the cells that is associated with numerous mitochondria (Fig. 5).

$Na^+ + K^+$ ATPase activity

For animals acclimated to 32 ppt salinity, whole-gill homogenates had ouabain-sensitive $Na^+ + K^+$ ATPase activities in the range of 35–65 nmol Pi mg pro^{-1} min^{-1}; activity in the coxal gland was significantly higher at about 120 nmol Pi mg pro^{-1} min^{-1} (Fig. 6). Moreover, while activity among whole-gill homogenates was variable, the distinctive anterior-posterior differences in $Na^+ + K^+$ ATPase activity seen in crustacean gills were clearly absent. Gill numbers 2 and 3 had significantly

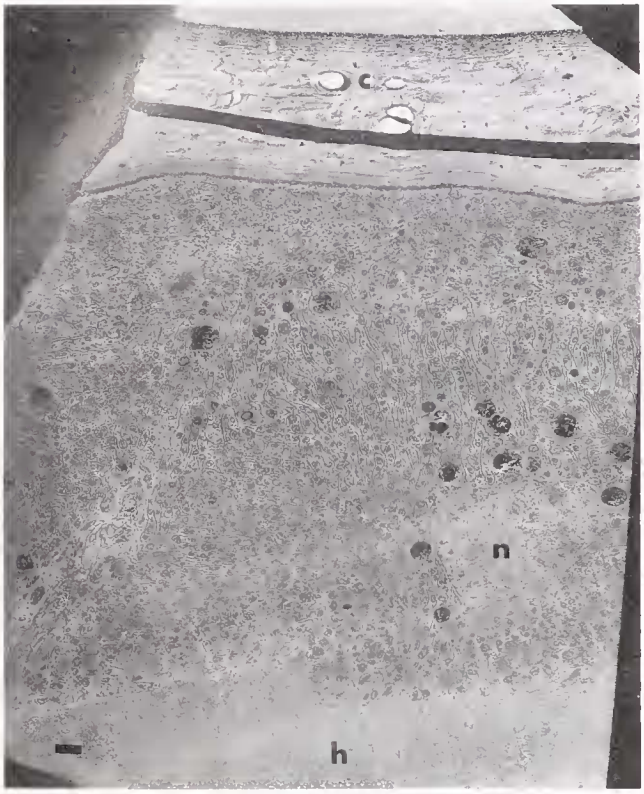


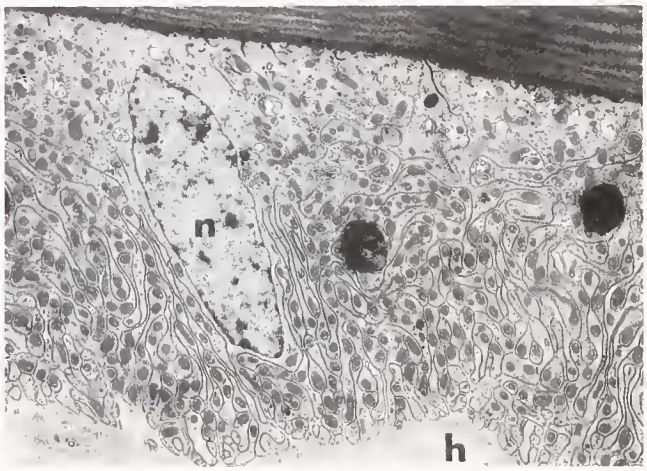
Figure 3. Semi-tangential section through a presumptive ion transporting cell in the ventral portion of the thick region. Note the elaborate membrane tubule network originating from infoldings of the basal membrane, the dense population of mitochondria, and the basal nucleus (n). Apical region of the cell is under the cuticle (c), and the basal region is in contact with the hemolymph (h). ×4515, scale bar = 1 μm.

higher activities than gills 1, 4, and 5. The values for the gills were about 2–3 fold higher than those reported by Towle *et al.* (1982), while activity in the coxal gland was 30% lower than reported previously for animals acclimated to comparable salinities.

The distribution of the $Na^+ + K^+$ ATPase activity within each individual gill, however, was highly localized. The central section of the lamellae contained the majority of the enzyme activity. $Na^+ + K^+$ ATPase activity in the central region (approximately 100 nmol Pi mg pro^{-1} min^{-1}) was about eightfold greater than that in the peripheral region (Fig. 6). With the exception of gill number five, there was no significant difference in $Na^+ + K^+$ ATPase activity within the central region in anterior *vs.* posterior gills. The relatively high variability of $Na^+ + K^+$ ATPase activity in whole-gill homogenates (*vs.* relatively low variability in the central region) might reflect the variability, from gill to gill, in the ratio of the mass of the central region to that of the whole gill. Activity in the peripheral region, however, was uniformly low throughout all gills. Further physiological differentiation was revealed when the dorsal and ventral epithelia were peeled apart and the individual layers examined. In the central but not the peripheral regions, $Na^+ + K^+$ ATPase activity was higher in the ventral portion of the epithelium. Acclimation to low salinity (15 ‰) for 36 h did not result in any increase in enzyme activity in either the gills or the coxal gland (data not shown); lack of short-term $Na^+ + K^+$ ATPase activation in *Limulus* gills was also reported by Towle *et al.* (1982).

Carbonic anhydrase activity

The distribution of CA in *Limulus* gills followed that of the Na/K ATPase, the pattern in most arthropod ion



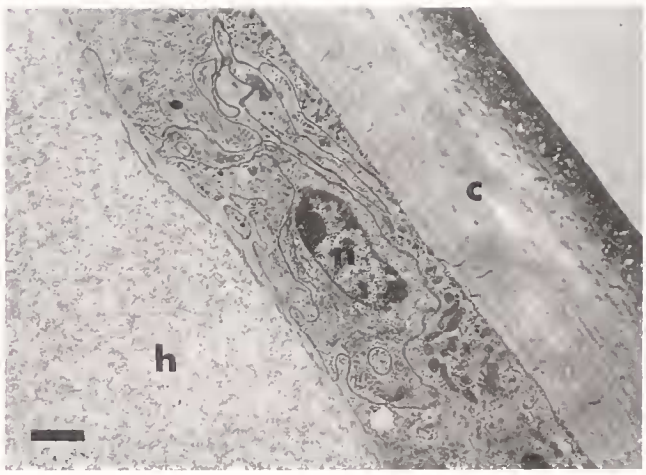


Figure 4. Comparison between presumptive ion transporting cells of the central ventral region of the gill (top) and presumptive respiratory epithelial cells of the peripheral region (bottom). The peripheral region lacks the extensive internal tubular system and the high density of mitochondria. ×7500, scale bar = 1 μm.

Figure 5. Tangential section through presumptive ion transporting cells of the coxal gland. Note the extensive internal tubular system (t) and numerous mitochondria (m). ×14,400, scale bar = 1 μm.

transport epithelia. In animals acclimated to 32 ‰, the level of CA activity among the five gill books was uniform (Fig. 7A). The uniform distribution and levels of activity (200–300 μmol CO_2 mg pro^{-1} min^{-1}) were both similar to those seen in euryhaline marine crustaceans (*e.g.*, Henry and Cameron, 1982a; Piller *et al.*, 1995). The central region of the lamellae also contained the bulk of the branchial CA activity; levels there were between 10 and 20 times greater than those in the peripheral region. Unlike the $Na^+ + K^+$ ATPase, however, CA activity in the coxal gland was low compared to that in the gill (Fig. 7A).

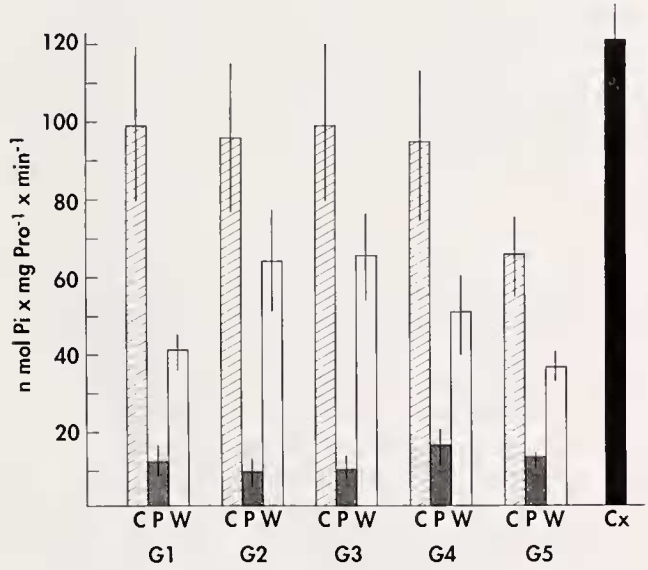


Figure 6. $Na^+ + K^+$ ATPase activity in homogenates of the five gill books (G1–5) and coxal gland (Cx) of *Limulus* acclimated to 32 ‰ salinity. Data for the gills are divided into whole-gill activity (W), and activity from the central (C) and peripheral (P) regions. Mean ± SEM (n = 5–6).

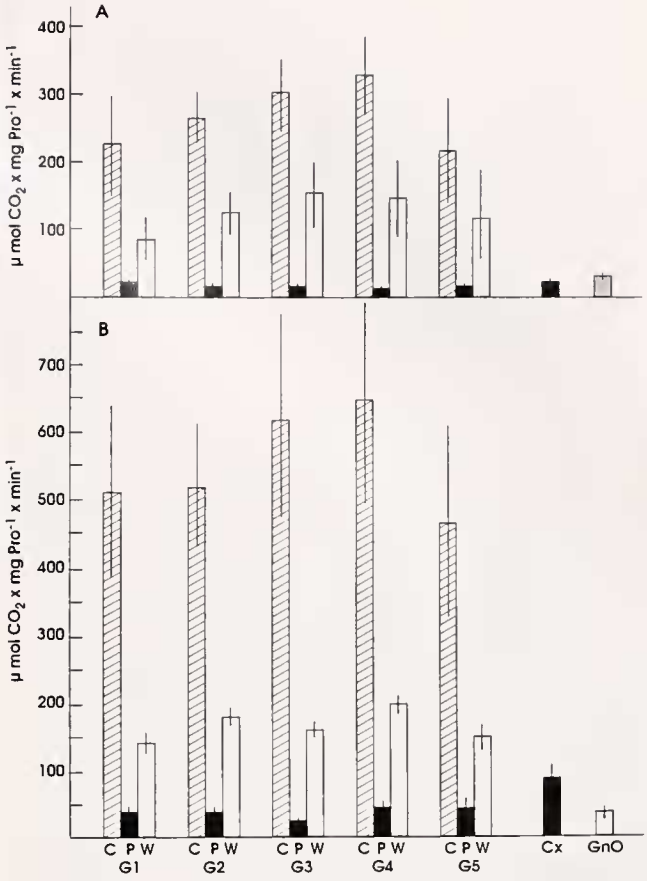


Figure 7. Carbonic anhydrase activity from the five gill books (G1–5), coxal gland (Cx) and gonadal operculum (GnO) of *Limulus* acclimated to 32 ‰ (A) and 10 ‰ (B) salinity. Gill activity is divided into whole-gill activity (W), and activity from the central (C) and peripheral (P) regions. Mean ± SEM, n = 6.

Long-term acclimation (2 weeks) to 10 ‰ salinity induced CA activity in all gills and in the coxal gland (Fig. 7B). Activity in whole-gill homogenates increased from 5 to 65% with virtually all of the induced CA activity occurring in the central region of the lamellae. While the CA activity in the peripheral region increased significantly, the absolute values were very small (*e.g.*, an increase of 12 μmol CO_2 mg pro^{-1} min^{-1} for gill 3). By comparison, the increases in the central regions of all gill books were very large (250 to 300 μmol CO_2 mg pro^{-1} min^{-1}). After low salinity acclimation, CA activity in whole gills doubled at best, and the increase occurred uniformly in all gills. CA activity in the coxal gland tripled in animals acclimated to 10 ‰, but it remained low in comparison with the gills.

Discussion

Despite being an osmotic and ionic regulator of moderate capacity, *Limulus* has a migratory pattern that brings it into estuarine waters of low salinity and shares this feature with euryhaline decapods that are strong regulators. *Limulus* is typically found in nature in salinities as low as 7–9 ‰ (McManus, 1969; Cavanaugh, 1975; Cohen and Brockman, 1983), but it has also been reported to survive in 2 ‰ in the laboratory (Warren and Pierce, 1982). Depending on acclimation salinity, *Limulus* maintains its hemolymph between 50 and 300 mosm Kg H_2O^{-1} above the ambient medium, which at best is only half the osmotic gradient that strong regulators (*e.g., C. sapidus*) can support. To survive in and exploit estuarine waters, *Limulus* appears to have independently evolved many of the same physiological and biochemical adaptations found in both euryhaline marine and freshwater arthropods.

Like many euryhaline and freshwater decapod crustaceans, the salt-transporting cells in *Limulus* are concentrated in a specific area within the gill lamellae; in this case it is the central region, and perhaps even more specifically the central ventral region of the lamellae of the book gills. In all species thus far examined, the region of the gill in which the salt-transporting cells (chloride cells) are localized also contains the highest levels of activity of both the Na-K ATPase and CA. *Limulus* differs from euryhaline decapods, however, in that both the distribution of chloride cells and corresponding enzyme activities are uniformly distributed among all gills. There is little or no anterior-posterior differentiation in the distribution of chloride cells, $Na^+ + K^+$ ATPase activity, and CA activity that is characteristic of other species (Aldridge and Cameron, 1979; Neufeld *et al.*, 1980; Henry and Cameron, 1982a; Holliday, 1985; Harris and Bayliss, 1988). In this way *Limulus* is more similar to freshwater crayfish, which also have a uniform distribution of both chloride cells (Dickson *et al.*, 1991) and ion transport-related enzyme activities (Wheatly and Henry, 1987). The gills of *Limulus* have only 10–50% of the Na/K ATPase activity as do those of strong ion regulators, such as *Callinectes sapidus* (and other decapod crustaceans; *e.g.*, 100–400 nmol Pi mg pro^{-1} min^{-1}) (Towle *et al.*, 1976; Neufeld *et al.*, 1980; Siebers *et al.*, 1982; Holliday, 1985). On the other hand, Na/K ATPase activity in the coxal gland is much higher than that in the antennal gland of both euryhaline marine and freshwater ion regulators (Towle, 1981; Wheatly and Henry, 1987).

Among euryhaline marine arthropods, *Limulus* appears to be unique in that its excretory organ, the coxal gland, has both the ultrastructural and biochemical characteristics of an ion transporting epithelium and also appears to play a clear role in hemolymph ion regulation (Mangum *et al.*, 1976; Towle *et al.*, 1982; also present findings). The present results show that CA activity is much higher than that found in the antennal gland of euryhaline decapods (*e.g.*, Henry and Cameron, 1982a), and that it is sensitive to salinity. In contrast, low and salinity-insensitive CA activity in the antennal gland of euryhaline decapods is believed to reflect the organ's primary role in the elimination of excess water; in fact, urine produced by the antennal gland is a major route of salt (NaCl) loss (Cameron and Batterton, 1978; Wheatly, 1985). Furthermore, $Na^+ + K^+$ ATPase activity in the coxal gland of *Limulus* is higher than that reported for the antennal gland of a moderately euryhaline crayfish (Wheatly and Henry, 1987); the antennal gland functions in the active reabsorption of Na and Cl and thus plays an important role in hemolymph ion regulation (Wheatly and Toop, 1989).

CA activities in the central region of the *Limulus* gill and in the coxal gland are salinity-sensitive and are induced by exposure to low salinity. The time course of induction, about two weeks, is similar to that found in both euryhaline marine and freshwater decapods (Henry and Cameron, 1982b; Henry and Wheatly, 1988). The degree of induction (twofold) was relatively small compared to the 8- to 10-fold changes in CA activity seen in the posterior gills of euryhaline crustaceans that are strong ion regulators (Henry and Cameron, 1982a; Piller *et al.*, 1995). There was no change in $Na^+ + K^+$ ATPase activity in either the gills or the coxal gland over a 36-h transfer from high to low salinity. This was also reported by Towle *et al.* (1982) and suggests that short-term activation of existing enzyme was not taking place. Activity was not monitored for a longer period.

Given the apparent ion-regulatory nature of the central region of the gill and the coxal gland, we suggest that the lower limit of salinity tolerance in *Limulus* may not be set by an inability to take up and conserve ions. Rather, the high water permeability of both the carapace and gills, and the resultant tissue swelling that accompanies hemolymph dilution in low salinity, may be the constraints that restrict *Limulus* from invading and permanently inhabiting waters of low salinity (<10 ‰). The water permeability of the carapace of *Limulus* is about tenfold higher than that in decapod crustaceans (Hannan and Evans, 1973; Dunson, 1984). In addition, the gills in *Limulus* are estimated conservatively at being fivefold more permeable to water than the carapace, while permeability to salts is relatively low (Dunson, 1984). Severe swelling in the gills has been reported for *Limulus* in low salinity, and this has been correlated with ballooning and hemorrhage as a possible cause of death (Mangum *et al.*, 1976; Dunson, 1984). Water gain, and not salt loss, is

probably the physiologically disruptive event in low salinities.

High water and low salt permeability would tend to establish a steep osmotic gradient between hemolymph and water in low salinity, and this would result in significant water gain by tissues. Heart tissue wet weight increases about 140% immediately upon transfer to low salinity, and interestingly, intracellular volume readjustment is slow and incomplete (Warren and Pierce, 1982). *Limulus* lacks the typical mechanism of rapid cell volume readjustment through a reduction of the intracellular free amino acid pool (Moran and Pierce, 1984); rather it relies on a biphasic mechanism of early intracellular ion loss followed by a much slower reduction in intracellular glycine betaine concentrations (Warren and Pierce, 1982). At extremely low salinities, this could limit the ability of *Limulus* tissues to reduce swelling; or the loss of intracellular K^+ beyond a critical point could cause enough damage to excitable tissues such as nerve and muscle to result in systemic failure. Severely swollen gills have indeed been reported for *Limulus* in low salinity (Mangum *et al.*, 1976; Dunson, 1984).

High water permeability has been proposed as an adaptation for the reproductive strategy of *Limulus*, as these high permeability values originate in the early developmental (egg and globe) stages (Laughlin, 1981). The egg cases are deposited intertidally in sand beaches and are therefore potentially subject to desiccation. High water permeability would ensure rapid rehydration upon contact with water, while low ion permeability would ensure conservation of salts in the face of exposure to freshwater runoff. Limitations in ion regulation and cell volume readjustment in the adult may have resulted from the selection for mechanisms of ensuring a rapid and constant state of hydration in the early developmental stages.

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